Analysis of the Metabolism of Dextran-Methylprednisolone Succinate

Using *In Vitro* Models

An Abstract of a Thesis by
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A brief review of past and present immunosuppressive treatments, including corticosteroids, cyclosporine A, and tacrolimus as they relate to liver transplantation is presented. The most common and detrimental side effects are presented for each therapy, and a method to reduce these adverse effects is proposed. As a method of drug delivery targeted to the liver, methylprednisolone is joined to a 70,000 Da dextran molecule through a succinic acid linker to form Dextran-methylprednisolone succinate (DexMPS). A newly developed reversed-phase HPLC assay is validated, and is capable of simultaneous measurement of methylprednisolone (MP), methylprednisolone succinate (MPS), methylprednisone (MPN), and corticosterone (CST) in addition to an internal standard (IS), triamcinolone acetonide. Ratios between drug plasma concentration and IS peak area were determined to be linear in the range of 0.1-4 μg/ml for MP and MPS, and 0.1-1.0 μg/ml for MPN and CST. Assay intra- and inter-run errors were less than 8%, demonstrating its accuracy and precision. The efficiency of the assay was determined by analysis of samples with and without the extraction steps. All components were at least 80% extracted. Application of the assay to *in vitro* hydrolysis experiments included DexMPS incubation in rat blood and phosphate buffer, and in rat liver lysosomes and various buffers. The half-life of DexMPS in blood was determined to be approximately 25 hours, and breakdown is most likely caused by chemical hydrolysis. The hydrolysis of MPS to MP is thought to be enzymatic, based on 10-fold faster breakdown in blood than in buffer. No significant hydrolysis was observed in isolated lysosomes for DexMPS or MPS. In addition, preliminary studies regarding rat spleen lymphocyte isolation and mitogen stimulation were performed. Optimum conditions for culture of spleen lymphocytes require RPMI-1640 to be supplemented with 2.5% rat serum. Separation of rat liver cells was accomplished by perfusion of the liver with HEPES buffer containing collagenase, followed by centrifugation through a density gradient of Percoll to yield populations of hepatocytes (93% pure, with 80% viability) and Kupffer cells (98% pure, with 92% viability). Together, these experiments provide a framework for examining the delivery of DexMPS to the liver, and measuring the effect of the DexMPS hydrolysis *in vivo*.

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by

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INTRODUCTION AND BACKGROUND

Organ transplants are a large part of modern medicine. The ability to replace one or more non-functional, diseased, or damaged organs with those from a donor demonstrates the triumph of man over death. A closer look at these life saving procedures reveals that the liver transplant is among the most complex and costly of all surgeries (1). In addition to the hospital charges for the initial surgery and associated treatments, a liver transplant recipient can expect bills to total $200,000 (2); much more if severe complications arise. Contrary to public opinion, there are many causes for liver transplants, including nonalcoholic cirrhosis, alcoholic cirrhosis, biliary atresia, tumors, among others (3).

Problems associated with organ transplantation are common. In fact, it is estimated that up to 50% of all transplants would result in rejection if untreated (4). The basis of this problem is simple: the recipient mounts an immune response against the graft and attempts to destroy the foreign tissue. This occurs when host lymphocytes, especially cytotoxic T cells, recognize various surface antigens on the graft tissue. These antigens, genetically encoded in the HLA complex, are referred to as the tissue type. Similar to blood typing, an ideal transplant would involve matched haplotypes. This rarely happens, as it requires a genetically identical donor and recipient. Even in the case of closely matched tissue types, rejection can occur when host T cells recognize minor histocompatibility markers. In short, the host's immunologic response against the graft involves a cell-mediated response with two major steps. First, those lymphocytes that are able to recognize the transplanted tissue as foreign or altered-self cells are stimulated to differentiate and reproduce. After this period of

sensitization, the mature cytotoxic lymphocytes attempt to destroy the foreign cells through directed release of perforins and granzymes (5), causing what is known as rejection. Acute, or cellular, rejection usually occurs within 4 weeks of the transplant, and affects up to 70% of all liver transplants (6). It is often reversible, especially if treated in the early stages. In contrast, chronic or ductopenic rejection is most often irreversible, requiring retransplantation. Fortunately, chronic rejection occurs in less than 16% of liver transplants (1). Interestingly, those patients who develop chronic rejection to primary transplants often battle it again in secondary and tertiary grafts (7).

After surgical techniques developed sufficiently to allow organ removal and replacement, and the mechanisms of the immune system became known, the need for the ability to suppress the immune response became apparent. During the 1970's the first widely accepted treatment consisted of corticosteroid therapy. Prednisone, often accompanied by azathioprine, was the most successful (7). However, this treatment was associated with a high incidence of rejection, poor graft survival, and poor patient survival. In addition, life threatening side effects were common. Early liver transplants demonstrated only a 30% one year survival rate (1).

Corticosteroids, including prednisone, work by inducing lysis of lymphocytes, especially immature cells in the thymus (5). In humans, the primary effect of corticosteroids is to cause a significant decrease in the number of circulating lymphocytes; Corticosteroids are also potent anti-inflammatory agents due to their ability to reduce the killing capabilities of macrophages and neutrophils (5). In addition, T cell activation is decreased by suppression

of class II MHC molecules on macrophages. Azathioprine is a mitotic inhibitor which diminishes lymphocyte proliferation by blocking synthesis of inosinic acid. Both B and T cells are affected (5).

Side effects of steroid therapy include hyperglycemia, insulin resistance due to a lowered number of insulin receptors, decreased muscle uptake of glucose, and osteoporosis (1). Adrenal cortisol production is suppressed, causing inhibition of longitudinal growth in children. Recent evidence suggests the donated liver's catabolic abilities play a part in growth suppression; problems associated with breakdown of methylprednisolone lead to increased exposure to the steroid and more growth inhibition. This problem can be overcome through treatment with recombinant human growth hormone, but this treatment requires daily injections, is costly, and doesn't always work (4).

A major breakthrough came in the early 1980's with the use of cyclosporine A. This drug works by blocking the interaction of calmodulin with calcium (8). The same study has shown it to inhibit IL-2 gene transcription and RNA synthesis, preventing the activation of T cells. When combined with corticosteroids, dramatic increases in patient and graft survival were seen, with the 1 year patient survival rate up to 70% (2). In fact, the triple drug cocktail of cyclosporine, prednisone, and azathioprine is commonly used as the standard against which other immunosuppressive regimens are measured (7). In the later part of the decade, advances in anesthetic technology, intensive care management, and prophylaxis against infection, along with new guidelines for patient selection and better organ preservation allowed another increase in survival rates. Today's 1 year survival rate approaches 90% (2). Unfortunately,

treatment with cyclosporine A still leads to the development of a variety of adverse effects. The most common ones include renal toxicity, hypertension, and diabetes mellitus (8).

The continuing search for an immunosuppressive agent without negative effects next led to tacrolimus. Although structurally different from cyclosporine, tacrolimus also affects calcium mobilization in the early steps of T cell activation (10). It is thought that these two drugs bind different proteins with a common enzymatic activity. Also known as FK506, tacrolimus has been shown to be as effective as cyclosporine A in preventing graft rejection when combined with a low dose of corticosteroids; only 1% of patients required retransplantation and 13% needed OKT3 (monoclonal antibody to the CD3 antigen of T cells) treatment versus 4% and 33% of cyclosporine treated patients (10). In addition, tacrolimus treatment is effective at concentrations 10 to 100 times lower than cyclosporine (9), and shows a lower incidence and severity of hypertension (7). When tacrolimus is used as the primary immunosuppressive agent, it shows slightly higher patient and graft survival when compared to cyclosporine in one study (10). However, increased nephrotoxicity and neurotoxicity suggest that this method of treatment is not ideal. Again, problems such as diabetes mellitus, gastrointestinal toxicity, insulin resistance, and graft rejection are common.

One possible benefit to use of tacrolimus is the potential to remove steroids from the long-term treatment. A steroid weaning study by Tzakis, et al. shows only 10% of patients using tacrolimus required corticosteroids 9 months after their transplant. In contrast, all but one of the patients treated with cyclosporine continued to use prednisone for the entire study period, 18 months.

The combination of tacrolimus with low doses of steroids was initially regarded with great enthusiasm. Lower steroid doses meant less inhibition of endogenous steroid production, and fewer incidences of hypertension leading to better quality of life for survivors. However, the high renal toxicity and the increase in patients developing diabetes mellitus has caused this drug to be used more often as a rescue treatment for acute rejection.

Other newly developed drugs are able to be used in this fashion also. Some of these include OKT3 combined with antilymphocyte globulin (11), mycophenolic acid (7), and rapamycin(9). These drugs are the last steps in treating rejection before retransplantation, and are considered only after intravenous bolus corticosteroid treatment has been shown to be ineffective.

These methods of treatment have greatly increased both patient and graft survival. In addition, the occurrence and severity of side effects has been decreased to some extent. But, for the thousands of people affected by them, adverse effects are still all too prevalent. Therefore, continued research in the field of immunosuppression is needed. Goals for future methods must include not only improved graft and patient survival, but also an absence or decrease in side effects.

One way to avoid many of these unwanted side effects would be to target the delivery of the immunosuppressive agent to the site of action. Research in this field has focused on colon-targeting in the past (12,13). Here, we propose a novel mechanism for liver-targeting.

Dextran Use

Dextrans are large glucose polymers which have been used for decades as blood

volume expanders (14). One important feature of dextrans is that they are able to be metabolized *in vivo* (15). Thus, an administered dose is gradually eliminated from the body. The mechanism of clearance is based on the size of the dextran (14-18). In the rat, dextrans with molecular weight of less than 20,000 Da are removed from circulating blood by the kidneys. The glomerular filtration apparatus removes these dextrans quickly and efficiently. At 40,000 Da the dextrans are too large for this mechanism to be effective; renal pores are blocked (16). As found by R. Mehvar, the primary method of clearance for this type of dextran is fluid-phase endocytosis in the liver and spleen; the hepatic accumulation of 70 kDa dextrans is substantial (14-18). Therefore, attaching an immunosuppressive drug to a dextran of that size could increase its delivery to the liver, and allow decreased doses with accompanying benefits such as reduced toxicity and other side effects. With this information, experiments were designed to test the following hypothesis:

> Dextran-methylprednisolone succinate (DexMPS) provides more sustained local immunosuppression than does methylprednisolone (MP).

MATERIALS AND METHODS

Methylprednisolone is currently available, and is commonly used conjugated with succinic acid (MPS) to make it soluble *in vivo*. This is helpful when synthesizing the DexMPS conjugate according to the method of McLeod (13). [See Figure 1, from (19).]

Development of an HPLC assay

The first step in this project was to design a procedure for the analysis of several corticosteroids. Existing methods did not allow for simultaneous measurement of MPS and

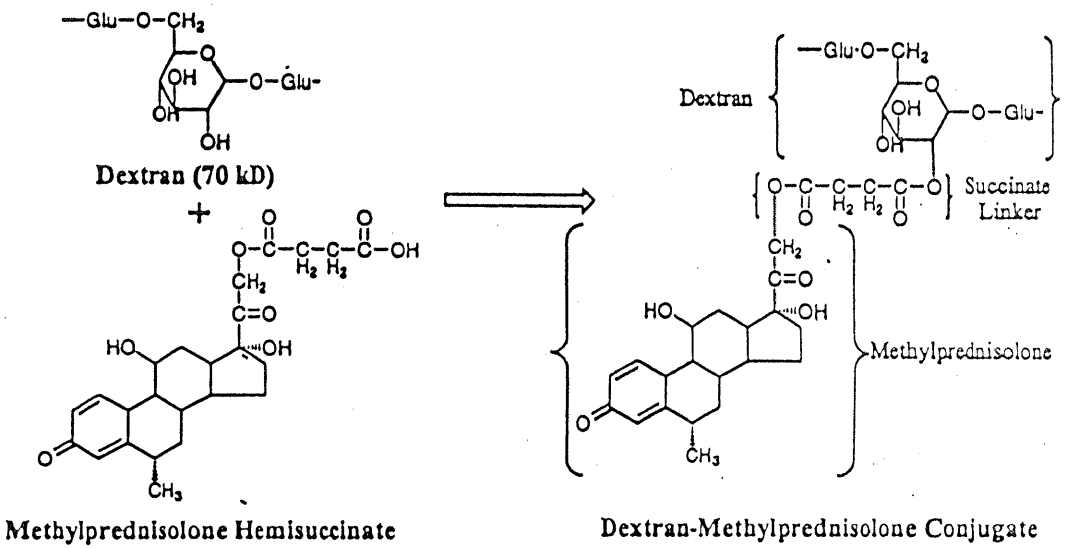


Figure 1. Structures of DexMPS, MPS, and MP.

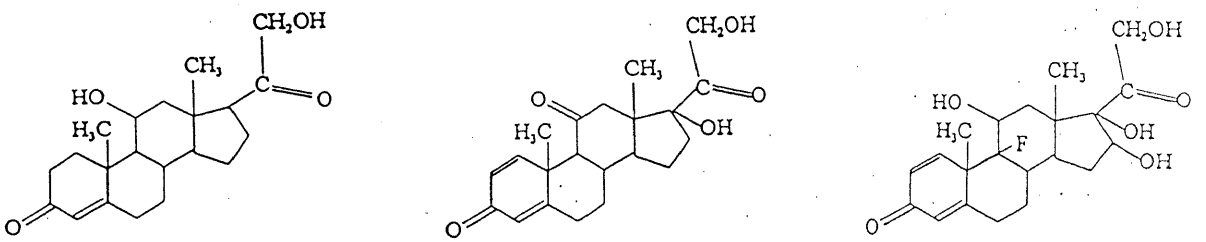


Figure 2. Structures of CST, MPN, and IS.

MP in the same sample, were not able to measure MP and its metabolite methylprednisone (MPN) with MPS, or did not allow determination of corticosterone (CST) with the other steroids (20-22). Refer to Figure 2 for structures. However, these published assays provided a pool of ideas from which we designed a working procedure. We also tested a variety of other corticosteroids to find a suitable internal standard. For this assay, we chose triamcinolone acetonide as the internal standard (IS). The final procedure follows:

In a 15 ml screw top test tube, 500 µl plasma and 100 µl IS (5 µg/ml in 10% acetic acid) were combined. Hexane (10 ml) was added, and samples were mixed on a rotary mixer for 10 minutes at 20 rpm. Next, the tubes were centrifuged for 3 minutes; the hexane (upper) layer was aspirated and discarded. Methylene chloride (8 ml) was added to the plasma, then tubes were mixed on the rotary mixer for 20 minutes at 20 rpm. Samples were centrifuged for 10 minutes, then the plasma (upper) layer was aspirated and discarded. The methylene chloride was decanted into a clean tube containing 100 µl glacial acetic acid, then evaporated to dryness under nitrogen flow in a 37°C water bath. The residue was dissolved in 200 µl mobile phase, then vortex mixed for 10 seconds. The liquid was transferred to a microfuge tube, centrifuged for 3 minutes, then the supernatant was transferred to a clean HPLC vial.

Samples were run on a 15 cm Hamilton PRP-1, 5 µm column with mobile phase of 24:76 acetonitrile:0.1 M sodium acetate, with flow rate of 1.5 ml/min. Steroids were detected at 250 nm using a Waters 484 UV detector. Other equipment used included a 590 pump (Waters), and a Sil9A auto sampler (Shimadzu). Chromatographic data was managed using Millenium software. A sample chromatogram is shown in Figure 3.

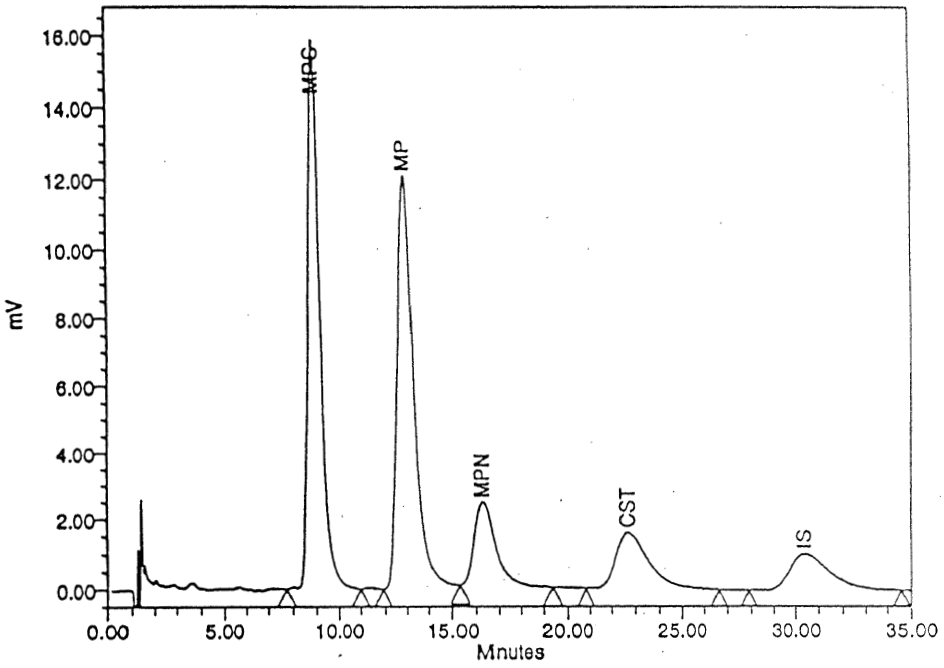


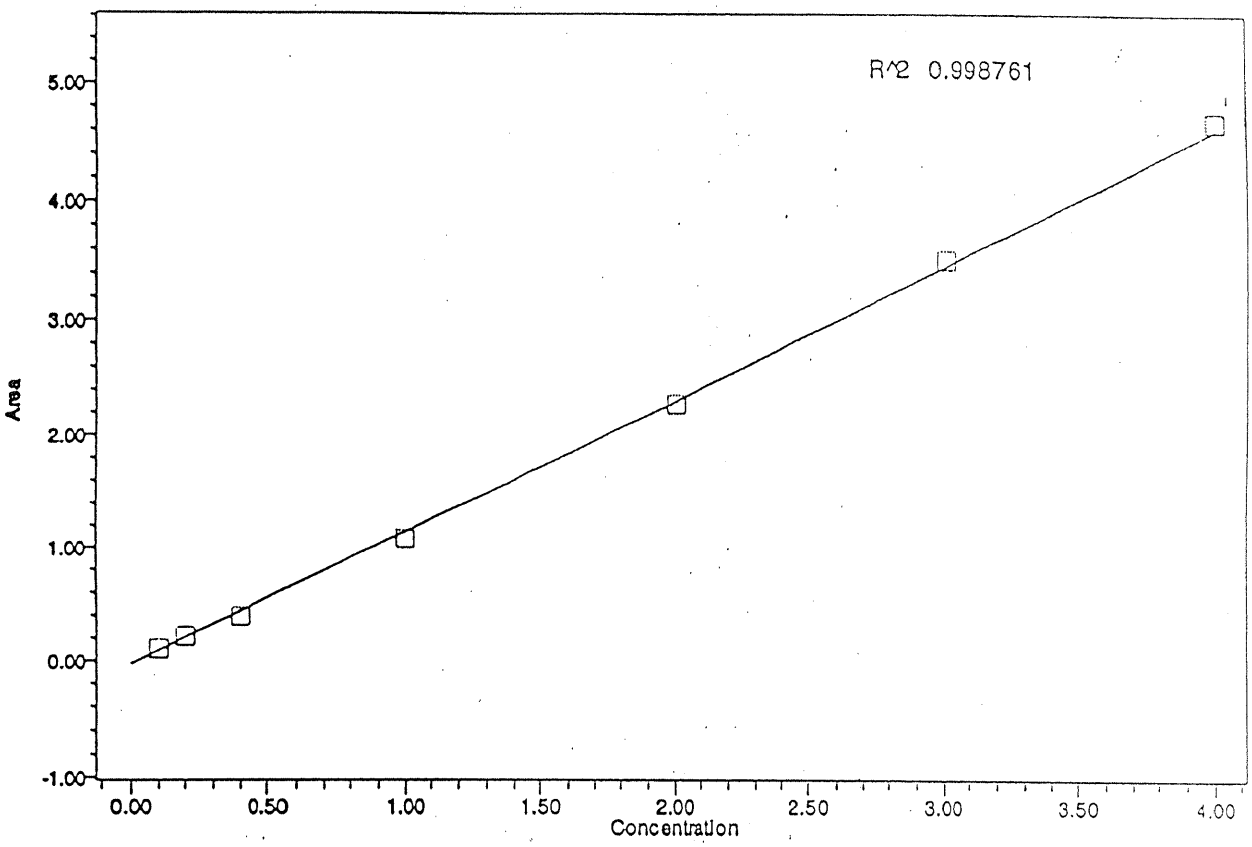
Figure 3. Example of Chromatogram for HPLC Assay.

Assay validation studies

This assay was tested for linearity of HPLC detector response compared to sample concentration. Accuracy, precision, and recovery were determined. Calibration curves of standard drug concentrations were prepared at concentrations of 0.1, 0.2, 0.4, 1, 2, 3, 4 μg/ml for MPS and MP. MPN and CST were included at one-fourth the concentrations of MPS and MP, and were quantified at 0.1, 0.25, 0.5, 0.75, and 1 μg/ml. All standards contained 100 μl IS at 5 μg/ml. Linear regressions for plots of detector response (area under the curve) vs predicted concentration were calculated for each drug. An example of a calibration curve is shown in Figure 4. Accuracy was determined from intrarun samples consisting of one calibration curve plus 5 each of standards 0.1, 0.4, 1, 4 μg/ml MPS that were run as samples. Precision was determined in a similar manner; 5 interrun sets consisting of one calibration curve plus one each of samples 0.1, 0.4, 1, 4 μg/ml. In a study of drug recovery, the ratio of peak height from processed MPS standards to unprocessed standards was compared.

Application of the HPLC assay

The first condition examined was the hydrolysis of MPS to MP. *In vitro* experiments were conducted with known concentrations of MPS that were incubated for 1 hour in a 37°C water bath. Samples of 1, 2.5, 5, 7.5, and 10 μg/ml MPS (MP equivalent) were incubated in either rat blood or isotonic phosphate buffer. One ml samples were taken at time 0, and after 1 hour. Blood samples were centrifuged immediately at 4°C, yielding plasma which was mixed with IS and frozen until analysis. Buffer samples were simply mixed with IS and frozen until analysis by HPLC. Hydrolyzed MP was plotted vs MPS concentration.



Calibration Level	Predicted Concentration (µg/ml)	Detector Response (µg/ml)	Calculated Value
1	0.10	0.1055	0.1115
2	0.20	0.2140	0.2049
3	0.40	0.3921	0.3585
4	1.00	1.0775	0.9480
5	2.00	2.2746	1.9781
6	3.00	3.5115	3.0426
7	4.00	4.6899	4.0567

Figure 4. Example of calibration curve for HPLC assay, generated using methylprednisolone succinate.

The next experiment involved the measurement of hydrolysis products of DexMPS. This *in vitro* procedure called for incubation of known amounts of DexMPS in pooled rat blood or isotonic phosphate buffer for 3 hours. Concentrations of 2.5, 5, 10, 25, 50, 75, and 100 μg/ml (MP equivalent) were made. Sample preparation and analysis were as above. Measurements of DexMPS concentration (17) and released MP and MPS in blood were made. Corresponding values obtained from incubation in buffer were also determined. The rate of MP and MPS production was calculated by subtracting the amount of MP or MPS present in the time 0 sample from that present in the 3 hour sample, then dividing by 3.

Another use of the HPLC assay was measurement of MP and MPS in frozen samples containing DexMPS. For this procedure, DexMPS was added to rat plasma at a concentration of 50 μg/ml MP equivalent. Triplicate samples were analyzed at time 0, and after 1, 3, 7, and 14 days of storage at -80°C.

A final check on the analytical procedure was to compare standards in rat plasma to those in phosphate buffer with albumin. Pooled rat plasma was cleaned with charcoal as per Haughey and Jusko (21). The slope of calibration curves for each drug were compared between the two systems; their similarity confirms that albumin is an acceptable substitute for rat plasma.

Isolation of lysosomes from rat liver

In this experiment differential centrifugation was used to extract lysosomes from a suspension of whole rat liver. Experiments similar to blood and buffer hydrolysis of MPS and DexMPS were then performed using the purified lysosomes. It was hypothesized that

increasing the concentration of lysosomal enzymes by incubating the drugs in isolated lysosomes would result in an increase in the rate of hydrolysis.

One of the keys to lysosome isolation is modification of their density. This is accomplished by the use of Triton WR-1339 (Tyloxapol). The procedure is taken from that designed by Trouet (23), with minor modifications. Once the liver fractions were isolated, their purity was checked with several enzyme assays. As reported by Trouet, the assays used include acid phosphatase activity for lysosomes, cytochrome oxidase activity for mitochondria, uricase activity for peroxisomes, and glucose-6-phosphatase for endoplasmic reticulum.

Male Sprague-Dawley rats (200-225 g) were injected i.p. with Triton WR-1339, at a dose of 85 mg/kg body weight. Four days later, after being fasted overnight, the rats were euthanized with CO_2. The livers were removed and weighed in cold 0.25 M sucrose, then cut into small pieces with scissors. Homogenization in 5 ml 0.25 M sucrose/g liver was completed using an electric hand-held homogenizer. This homogenate was centrifuged at 2645 rpm for 10 minutes (6500 g-min) at 4° C in a Sorvall Suprafuge RC2. The supernatants were aspirated and saved, then the pellets were resuspended in 0.25 M sucrose (3 ml/g liver). This mixture was centrifuged at 2300 rpm for 10 minutes (5000 g-min) at 4° C in the same centrifuge. After combining the supernatant with that from the previous centrifugation, it was diluted to a total of 10 ml/g liver, using 0.25 M sucrose. This liquid was the cytoplasmic extract, Fraction E. The pellets were suspended in approximately 50 ml 0.25 M sucrose and composed Fraction N, containing nuclei and cell debris.

Fraction E was then centrifuged at 27,000 rpm for 10 minutes (340,000 g-min), at 4° C in a Beckman L5-65 ultracentrifuge with a Ti70 rotor. As the supernatant was aspirated, the pink fluffy layer covering the pellet floated off and was removed. The pellets were then washed by resuspension in 5 ml 0.25 M sucrose/g liver followed by centrifugation at 27,000 rpm for 10 minutes. The supernatant and pink layer was combined with that from the previous step, and the volume measured. This was the PS fraction, which contained microsomes and cell sap.

The pellets were suspended in 1 ml 45% sucrose/g liver. This mixture was placed into tubes for the Beckman SW28 rotor, and covered with 22 ml 34.5% sucrose. Next, 14.3% sucrose was layered on top to within 2 mm of the top of the tube. The balanced tubes were then centrifuged at 24,000 rpm for 2 hours. During this time, the lysosomes floated to form a band between the 34.5% sucrose and the 14.3% sucrose layers. This band, Fraction T, was harvested using a Pasteur pipette. The remainder of the gradient and pellet was homogenized, yielding Fraction R which was made up of mitochondria and peroxisomes. For procedure overview, see Figure 5.

Fraction Assays

Protein Analysis

The protein content of each fraction was determined using the Bradford method. One hundred µl of diluted tissue fraction was added to 1 ml dye concentrate plus 4 ml water. The absorbance at 595 nm was taken and converted to µg protein/ml using a standard curve based on bovine albumin.

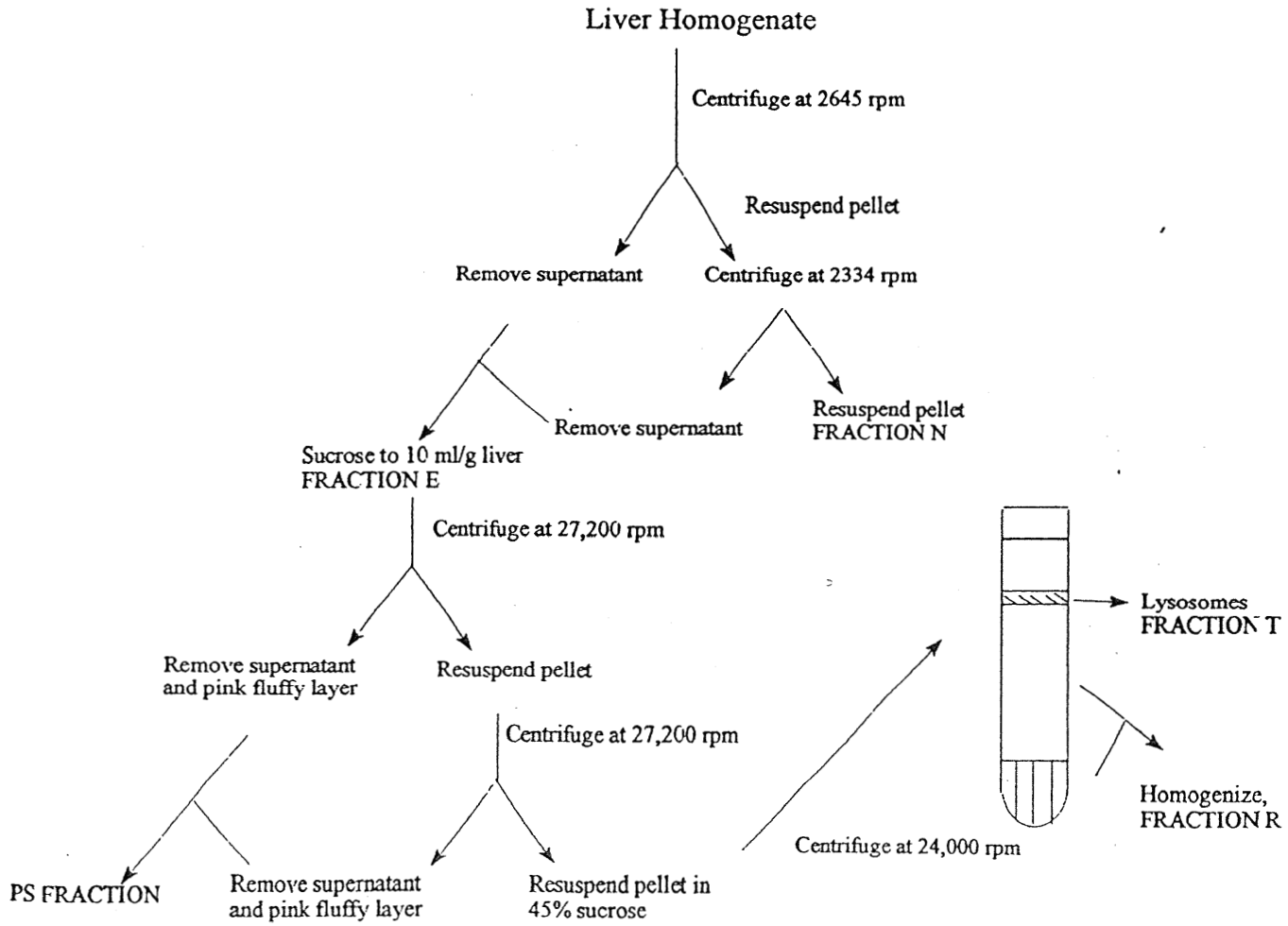


Figure 5. Scheme for lysosome isolation.

Acid Phosphatase

The acid phosphatase assay used was that reported by Trouet (23), with minor modifications. Rather than the 1 ml used by Trouet, 0.5 ml tissue fraction was incubated with 0.2 ml β-glycerophosphate, 0.1 ml 2% w/v Triton X-100, and 1.1 ml water for 30 minutes in a 37°C water bath. At that time, the reaction was stopped with 10 ml 8% TCA. In the case of blanks used to determine nonspecific phosphatase activity, the β-glycerophosphate was added after the TCA. The denatured proteins were sedimented by centrifucation (5000 rpm, 10 minutes). One ml of the supernatant was added to 1 ml 2.5% w/v ammonium molybdate in 5N H_2SO_4, 0.2 ml Fiske-Subbarow reducer (SIGMA chemical company), and 7.8 ml water. After 10 minutes, the A_{660} was read, allowing the μmoles of phosphate released to be determined as compared to a standard curve.

Cytochrome oxidase

The method employed by Trouet (23) was used for this assay. A standard solution of cytochrome c was reduced completely with sodium hydrosulfite. Three ml of this solution were placed in a 1 cm quartz cuvette with 20 μl of tissue fraction. The absorbance at 550 nm was read every 30 seconds for 5 minutes. Next, a few grains of potassium ferricyanide were added to the cuvette to completely oxidize the remaining cytochrome c. The A_{550} of the oxidized solution was subtracted from the earlier readings, then the μmoles of cytochrome c oxidized per minute was calculated for each fraction using a standard curve.

Uricase

Urate oxidase activity was measured in the same manner as by Trouet (23). In this

assay, 25-100 µl of tissue fraction was added to 0.2 ml potassium phosphate buffer (0.5M) and water to make 3.0 ml. The A_{292} of this mixture was read as a blank, then 10 µl of uric acid solution was added. The A_{292} was read in 1 minute intervals for 10 minutes. These values were converted to µmoles of uric acid oxidized per minute for each fraction as determined by a standard curve.

Glucose-6-phosphatase

Phosphatase activity at non-acidic pH was determined as reported by Trouet (23). Using a calibrated glass centrifuge tube, 0.1 ml of the substrate (glucose-6-phosphate) was mixed with 0.3 ml maleic acid buffer. This was brought to 37°C in a water bath, then 0.1 ml tissue fraction was added. This was incubated for 15 minutes in the water bath. The reaction was stopped with 1 ml 8% TCA. Next the tubes were chilled in ice for 5 minutes, diluted to 2.5 ml, and centrifuged at 5000 rpm for 10 minutes. One ml of the supernatant was taken for phosphate analysis as described above.

Isolation of lymphocytes from rat spleen and mitogen stimulation assay

Lymphocyte Isolation

The basic procedure followed was obtained from the SIGMA diagnostic procedure bulletin number 95 (24), which was designed for the isolation of human blood lymphocytes. Only minor modifications, such as use of a suspension of spleen tissue rather than a dilution of a whole blood sample and the use of RPMI-complete containing varying concentrations of rat serum rather than RPMI-1640 for the final cell dilution, were needed to adapt it for use with rat spleenocytes.

This procedure uses a density gradient material, Histopaque (SIGMA chemical company), to separate lymphocytes from other cell types. In the case of human lymphocytes, a fresh blood sample (3ml) was mixed with RPMI-1640 (5ml) then layered onto Histopaque (3ml). During centrifugation, a layer of lymphocytes formed which was aspirated off, washed with RPMI-1640, then recentrifuged. The resulting cell pellet was suspended in RPMI-1640, diluted to the desired cell concentration and assayed for viability using trypan blue.

Rat spleenocytes were isolated in a similar manner. The excised spleen was placed in HEPES buffered RPMI-1640 (25) and the tissue was disrupted using a stainless steel tissue grinder. The cells were collected in, and diluted with, 25 ml RPMI-1640. Eight ml of the diluted spleenocyte suspension were layered onto 3 ml of Histopaque-1077 in 15 ml plastic conical centrifuge tubes. The tubes were centrifuged at 400g (1500 rpm) at room temperature for 30 minutes using a swinging bucket type centrifuge (Damon IEC HM-SII). The upper layer of supernatant was aspirated and discarded, using a sterile pipette tip, to within 0.3 cm of the white band containing lymphocytes. This layer was transferred to a clean 15 ml centrifuge tube and mixed with 10 ml of RPMI-1640. This mixture was centrifuged at 1500 rpm for 10 minutes at room temperature. Following removal of the supernatant by aspiration, the cell pellet was suspended in 1 ml of RPMI containing the appropriate concentration and type of serum, 2mM Glutamine, and 1% penicillin/streptomycin (26), then diluted to a final cell concentration of $2x10^6$ cells/ml. During the process of counting and diluting the cells, viability was checked using the trypan blue exclusion assay.

Mitogen stimulation assay

In addition to studies of human peripheral blood lymphocytes and rat spleen lymphocytes, experiments were done to determine the effects of using RPMI supplemented with 10% fetal calf serum, 2.5% rat serum, or 5% rat serum on the proliferation of rat lymphocytes. Rat serum was obtained from blood collected through cardiac puncture of ether anesthetized rats. Fetal calf serum was obtained from Diamond Labs (Des Moines, IA).

One hundred µl of the cell suspensions were placed into the wells of a 96 well, flat bottom microtiter plate (Corning). An equal volume of mitogen was added in concentrations ranging from 0 to 20 µg/ml (27). Preliminary studies with human lymphocytes used phytohemagglutinin (PHA) and concanavalin A (Con A) (26). The rat lymphocyte studies used only Con A. The plates were then incubated in a 5% CO_2 atmosphere (Forma Instruments) at 37° C for 48 hours. Two µl of ^{3}H-thymidine (1.000 mCi/ml, New England Nuclear Supplies) were added to each well, and the plate was incubated for an additional 18-20 hours (25).

Following incubation, the lymphocytes were collected onto glass fiber filtermats using a Skatron Cell Harvester. The filtermats were dried at room temperature for 40-45 minutes, then the sample disks were placed into plastic scintillation vials. Scintillation fluid (3 ml) was added to each vial, which was then capped for storage.

Sample radioactivity was determined with 3 days of cell harvest using a Beckman LS 1800 Liquid Scintillation Counter. The resulting counts per minute (cpm) were averaged for the replicate wells and plotted using Microsoft Excel as cpm v. mitogen concentration.

Liver cell isolation

One method of analyzing the local effect of DexMPS on the liver is to examine the availability of DexMPS in liver endothelial cells and Kupffer cells. Concentrations of the drug can be measured through HPLC analysis, allowing conclusions to be drawn regarding storage and breakdown of DexMPS, and its availability to lymphocytes.

This procedure involved isolating and cannulating a rat liver as described by Seglen (28), with some modifications as proposed by Pertoft (29). The liver was perfused for 10 minutes with calcium/collagenase free HEPES buffer, 50 ml/min in a single pass system. Perfusion with HEPES buffer with 0.7 g $CaCl_2 2H_2O$/L and 0.5 g collagenase/L in a recirculating manner followed. After 10 minutes, or when the liver began to fall apart, the perfusion was stopped. The liver cells were dispersed in a sterile plastic petri dish containing 15 ml calcium free HEPES buffer with 1.5% BSA and 80 µg/ml DNAase. This suspension was passed through a double layer fine-weave nylon mesh which was then washed with 335 ml HEPES buffer with albumin and DNAase. The diluted cells were centrifuged at 70g (660 rpm) for 2 minutes at room temperature (Heraus Sepatech 13.92 rotor). The supernatant, containing nonparenchymal (Kupffer) cells, was aspirated and pelleted at 400g (1570 rpm). The original pellet of liver endothelial cells was washed twice in PBS. The washed LECs were suspended in PBS, then layered onto 16 ml 70% Percoll (SIGMA chemical company). This preparation was centrifuged at 800g (2300 rpm) for 10 minutes at room temperature, also in the HS 13.92 rotor. Contaminating NPCs and dead LECs remain on top of the Percoll, allowing a pellet of hepatocytes to be harvested. See Figure 6 for clarification.

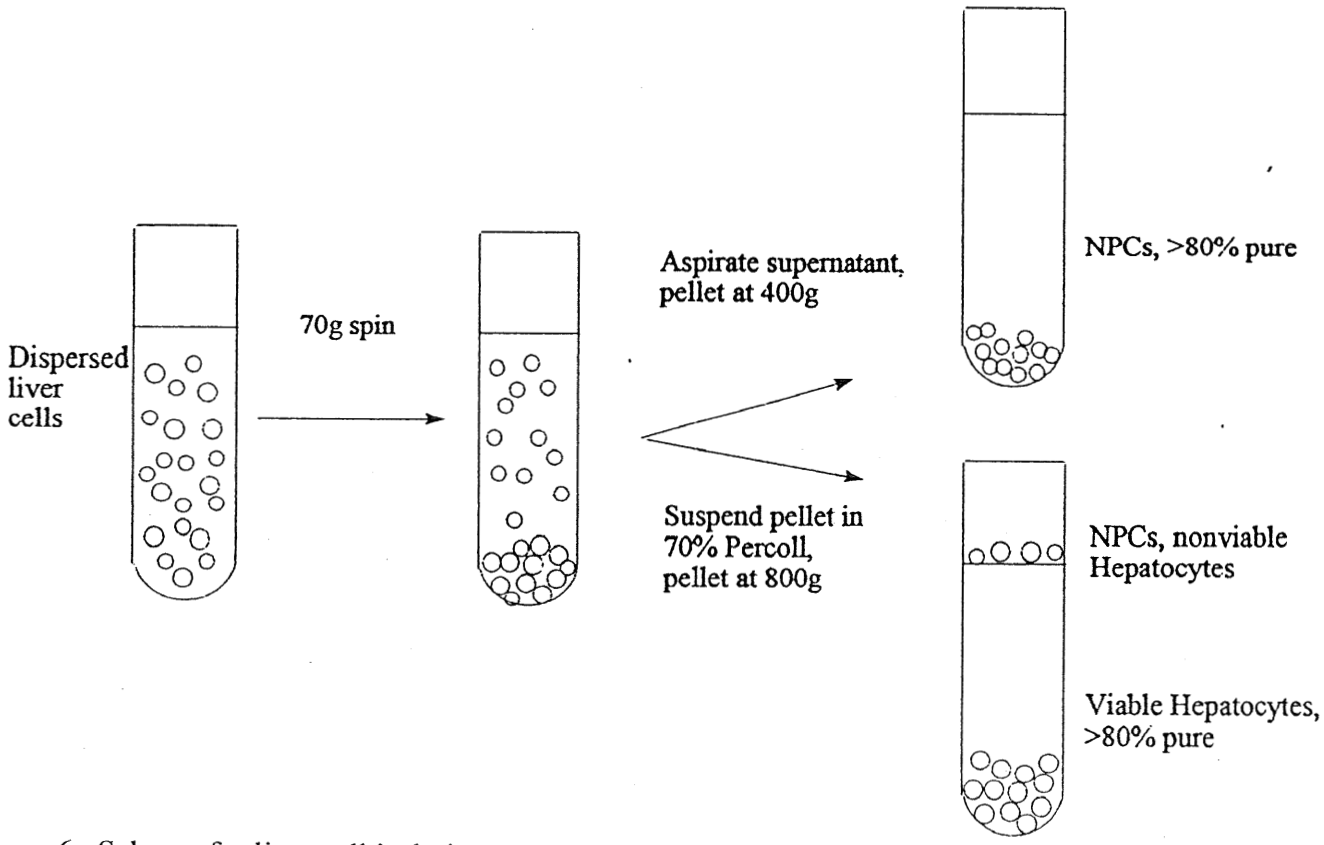


Figure 6. Scheme for liver cell isolation.

Determination of cell purity and viability

The determination of cell counts, purity, and viability was accomplished with a hemacytometer and trypan blue. Concentrated cell pellets were suspended in RPMI 1640, then 50 µl of the cell suspension was mixed with 50 µl trypan blue. Viable cells remained clear, dead cells were tinted blue. Cells were counted using the grids provided on the hemacytometer slide, then concentrations were calculated using the formula

$$(\text{Avg \# of cells} / 4 \text{ grids}) \times 2 \times 10^4 = \text{cells/ml}$$

where 2 is the dilution factor caused by trypan blue, and 10^4 is the factor of the hemacytometer slide which contains a volume of 0.1 ml^3.

RESULTS

Development and validation of an HPLC assay

The HPLC assay developed is capable of simultaneous measurement of 4 corticosteroids. All 5 assay components were baseline separated with little retention time variability observed. The limit of quantitation is 0.1 µg/ml for all drugs. Accuracy and precision are within acceptable ranges (Tables 1 and 2), and recovery of the steroids during the extraction procedure exceeds 80% for all drugs (Table 3).

Application of the HPLC assay

The slope of the trend line for MPS hydrolysis to MP (Figure 7) provides the rate constant of that reaction since it was allowed to proceed for one hour. However, a similar approach to DexMPS hydrolysis (Figure 8) does not provide the correct rates due to the

Table 1. Intrarun validation study data.

	MP	MPS	MPN	CST
	0.1 µg/ml			
Mean	0.0944	0.1070	0.1070	0.0960
Standard Deviation	0.0050	0.0172	0.0106	0.0126
Coefficient of Variation	5.3283	16.065	9.9567	13.156
Percent Error	-5.60	7.00	7.00	-4.00
	0.4 µg/ml		**0.25 µg/ml**	
Mean	0.4035	0.4110	0.2595	0.2335
Standard Deviation	0.2288	0.0502	0.0110	0.0177
Coefficient of Variation	5.6713	12.222	4.2506	7.6010
Percent Error	0.875	2.75	3.80	-6.60
	1 µg/ml			
Mean	1.0498	1.0303	1.0165	1.0393
Standard Deviation	0.0385	0.0488	0.0451	0.0432
Coefficient of Variation	3.6708	4.7388	4.4335	4.1554
Percent Error	4.98	3.03	1.65	3.93
	4 µg/ml			
Mean	4.1326	4.0622		
Standard Deviation	0.1495	0.1513		
Coefficient of Variation	3.6187	3.7239		
Percent Error	3.32	1.56		

Table 2. Interrun validation study data.

	MP	MPS	MPN	CST
	0.1 µg/ml			
Mean	0.1032	0.1024	0.1036	0.1070
Standard Deviation	0.0068	0.0095	0.0061	0.0099
Coefficient of Variation	6.5863	9.2799	5.8951	9.2282
Percent Error	3.20	2.40	3.60	7.00
	0.4 µg/ml		**0.25 µg/ml**	
Mean	0.3990	0.3966	0.2420	0.2310
Standard Deviation	0.0035	0.0103	0.0044	0.0163
Coefficient of Variation	0.8861	2.6058	1.8176	7.0405
Percent Error	-0.25	-0.85	-2.32	-7.60
	1 µg/ml			
Mean	0.9848	0.9760	0.9440	0.9616
Standard Deviation	0.0412	0.0454	0.0484	0.0471
Coefficient of Variation	4.1833	4.6565	5.1232	4.8958
Percent Error	-1.52	-2.40	-5.60	-3.84
	4 µg/ml			
Mean	3.8506	3.7936		
Standard Deviation	0.1385	0.2621		
Coefficient of Variation	3.5958	6.9089		
Percent Error	-3.74	-5.16		

Table 3. Recovery of HPLC Standards.

Average area units for 4 components

	MP	MPS	MPN	CST
Unprocessed	130366	131803	32205	36128
Processed	109328	117951	30675	32147
Ratio*100%	**90.48**	**82.95**	**95.25**	**86.49**

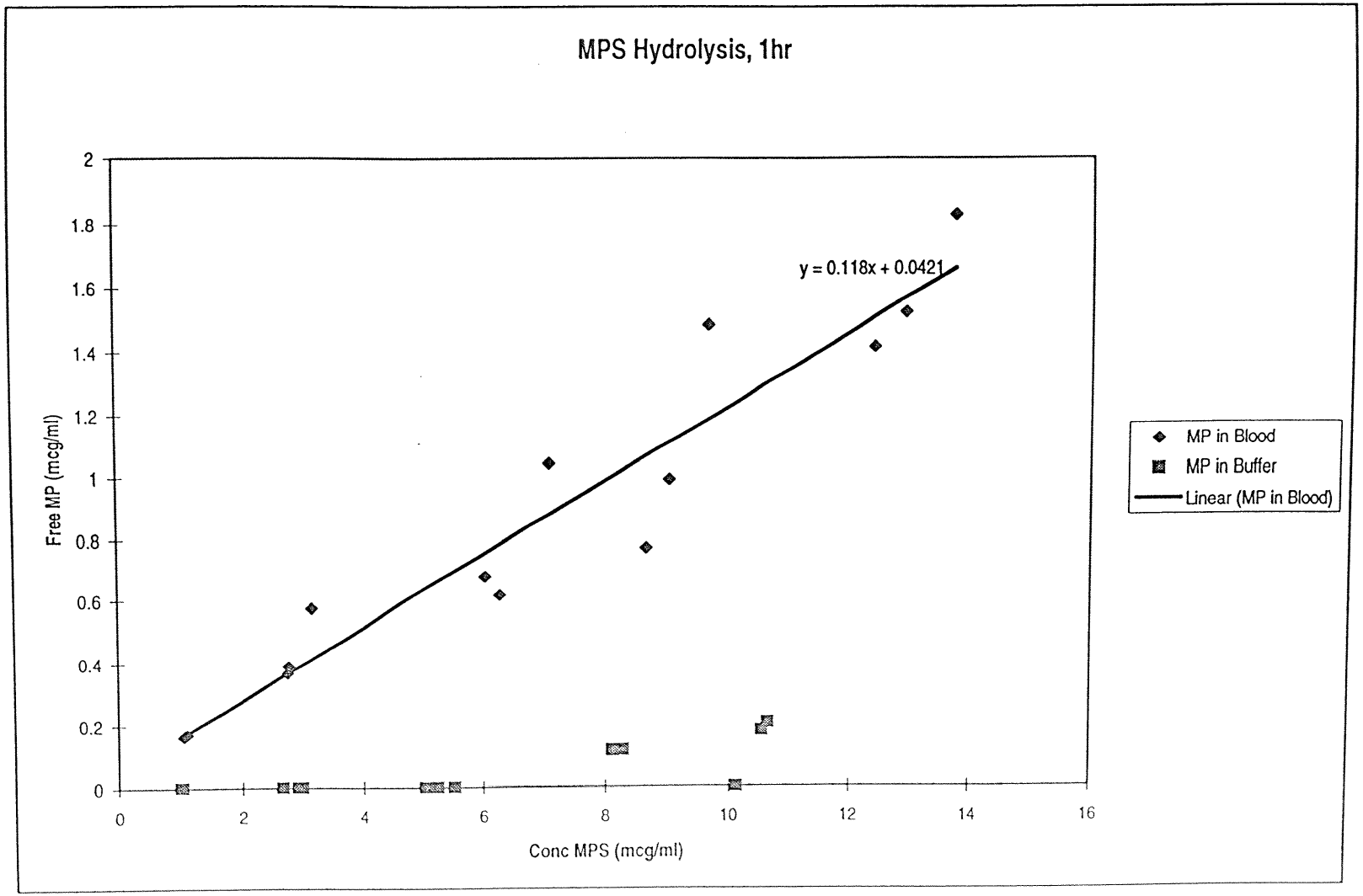


Figure 7. MPS hydrolysis in rat blood or phosphate buffer.

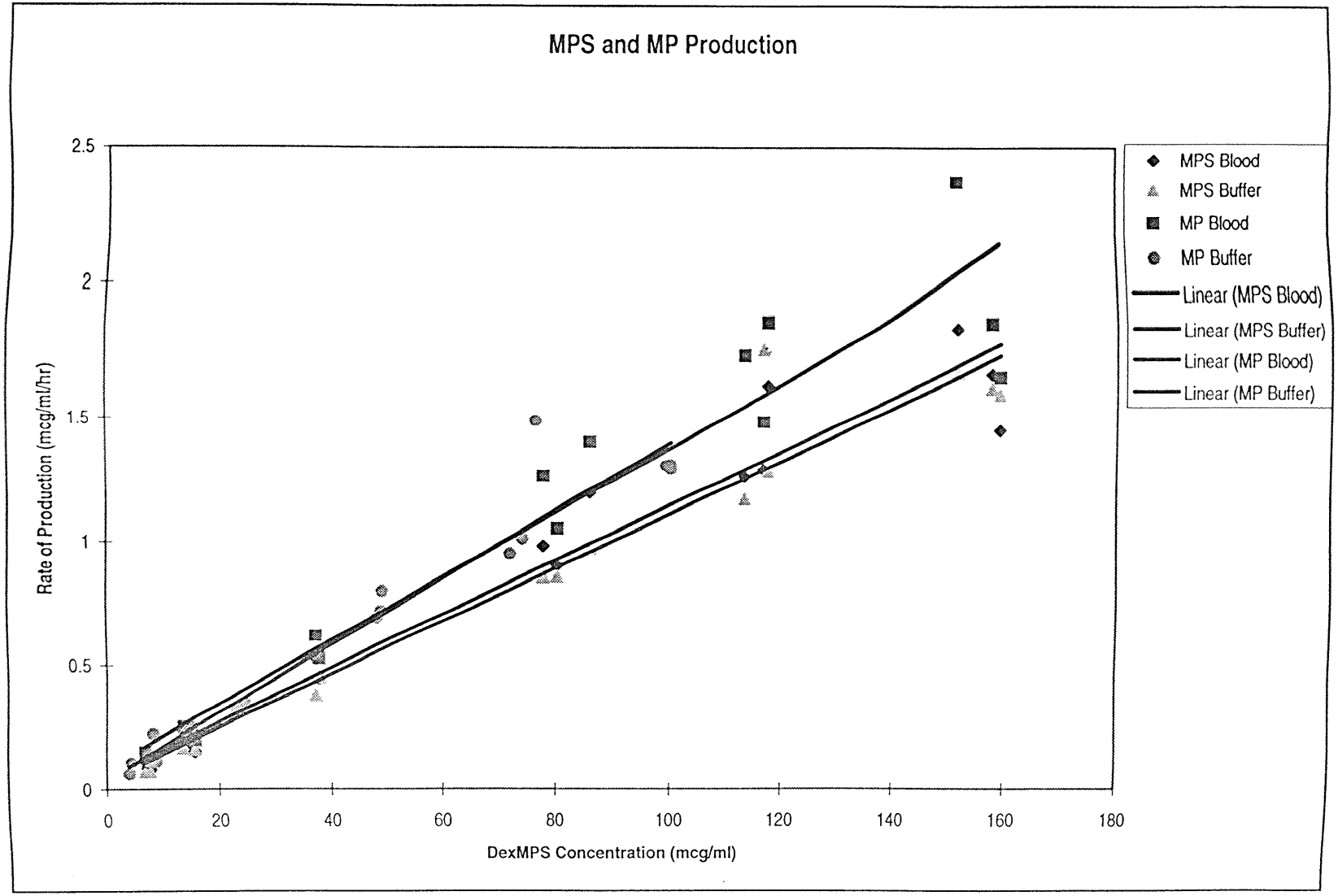


Figure 8. DexMPS hydrolysis (3 hour) in rat blood or phosphate buffer.

capability of released MPS to hydrolyze to MP. Using the known rate of MPS hydrolysis, measured concentrations of DexMPS at time 0, and measured concentrations of MPS and MP after 3 hours of incubation, rate constants for DexMPS hydrolysis to MPS and MP can be estimated (Table 4). Also shown in Table 4 are estimated half-lives of MPS and DexMPS in blood. These values were obtained using the equation:

$$t_{1/2} = 0.693/\text{rate}.$$

As expected, the half-life for DexMPS is substantially longer than that of MPS, due to the lack of dextranases in circulation, and the steric interference of the bulky dextran side chains with blood esterases. In the same manner, the release of MPS and MP from DexMPS occurs at nearly the same rate; they are cleaved by the same esterase acting at different ends of the succinic acid linker.

The study of *ex vivo* hydrolysis in frozen samples shows constant levels of MPS, MP, and CST over the sampling period (Table 5). This indicates that collected samples can be acidified by addition of IS solution, then frozen for up to two weeks before analysis.

Isolation of lymphocytes from rat spleen and mitogen stimulation

All cell samples (rat and human) were determined to be greater than 95% viable after isolation, as judged by trypan blue exclusion. The first trial using human lymphocytes stimulated with Con A and PHA was successful in obtaining a dose-response curve, so further trials were not undertaken. Additional trials were conducted using rat spleen lymphocytes cultured in RPMI containing 10% FCS or varying amounts of rat serum.

Table 4. Drug hydrolysis and half-lives in rat blood.

Reactant/Product	Rate Constant of Hydrolysis	Half-life in hours
MPS/MP	0.118/hr	5.9
DexMPS/MPS	0.0413/hr	48.5
DexMPS/MP	0.0135/hr	51.3

Table 5. HPLC analysis of DexMPS samples in rat blood. Average *ex vivo* hydrolysis of three frozen samples (μg/ml).

	MPS	MP	CST
0 Day	0.018	0.066	0.366
1 Day	0.018	0.121	0.383
3 Days	0.0	0.056	0.365
14 Days	0.084	0.0	0.439
Mean	0.034	0.056	0.391
Standard Deviation	0.049	0.051	0.046

Liver cell isolation

Low speed centrifugation is capable of pelleting the relatively large hepatocytes. Contamination of the supernatant containing Kupffer cells is low (see Table 6). Use of a single layer density gradient (70% Percoll) provides a quick, yet effective method of removing unwanted nonparenchymal cells and many dead hepatocytes from the parenchymal cell pellet.

Isolation of lymphocytes from rat liver and mitogen stimulation assay

The two assays for human lymphocyte proliferation using Con A showed dose response curves with peak mitogenic activity occurring when the concentration of Con A was 10 μg/ml (see Figure 9). Trials using rat spleenocytes cultured in 10% FCS exhibited little proliferation (see Figure 10). The rat spleenocyte proliferation in rat serum exhibited peak growth when Con A at a concentration of 2.5 μg/ ml was present in the wells (see Figure 11).

DISCUSSION AND CONCLUSIONS

HPLC assay

This novel HPLC assay is useful for both *in vitro* and *in vivo* experiments. The extraction procedure is efficient enough to allow quantification of all four components in a range of concentrations. In addition, this procedure is unique in its ability to measure MP, its precursor MPS, and one metabolite, MPN. Thus, it is of potential use for other researchers examining the *in vivo* breakdown of corticosteroids.

Isolation of lysosomes from rat liver

The method of isolation described by Trouet has been thoroughly tested since it was first proposed. Never the less, we double checked our fractions using the proposed enzyme

Table 6. Liver cell isolation: Kupffer cells and hepatocytes.

	Hepatocytes	Viability	Kupffer cells	Viability
Original suspension	72%	86%	28%	96%
Kupffer cell pellet	2%	100%	98%	92%
Washed hepatocytes	93%	80%	7%	80%

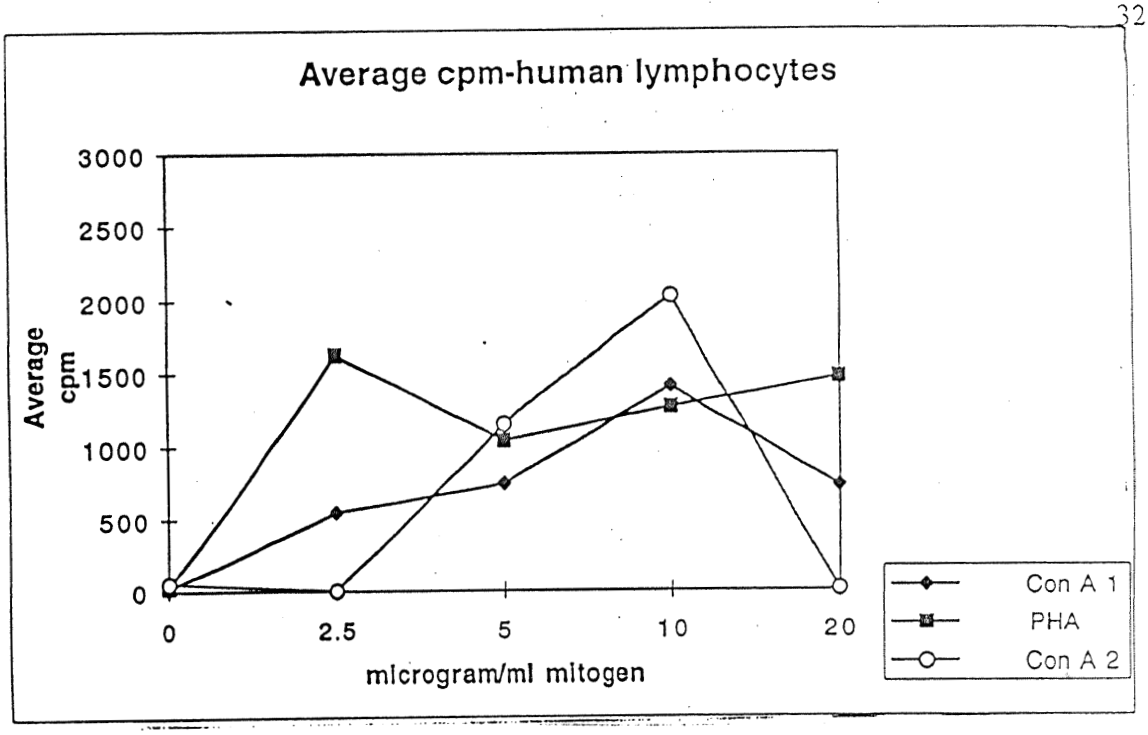


Figure 9. Human blood lymphocyte stimulation, cells cultured in RPMI-1640 supplemented with 10% fetal calf serum.

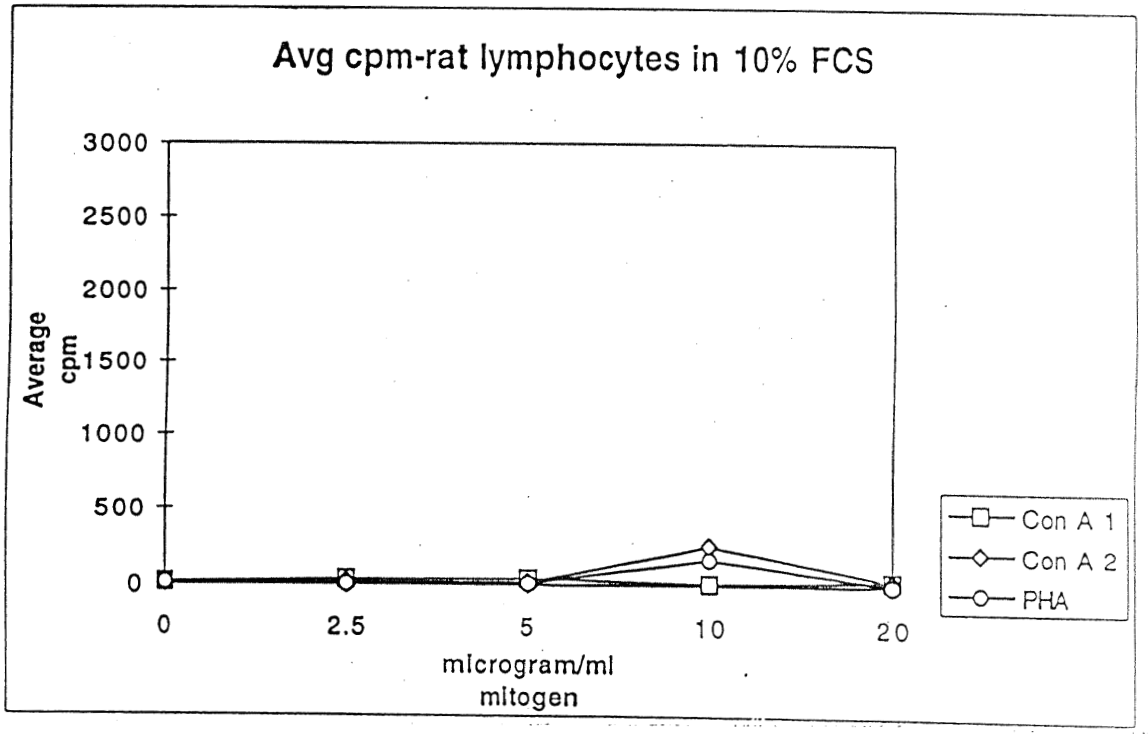


Figure 10. Rat spleen lymphocyte stimulation, cells cultured in RPMI-1640 supplemented with 10% fetal calf serum.

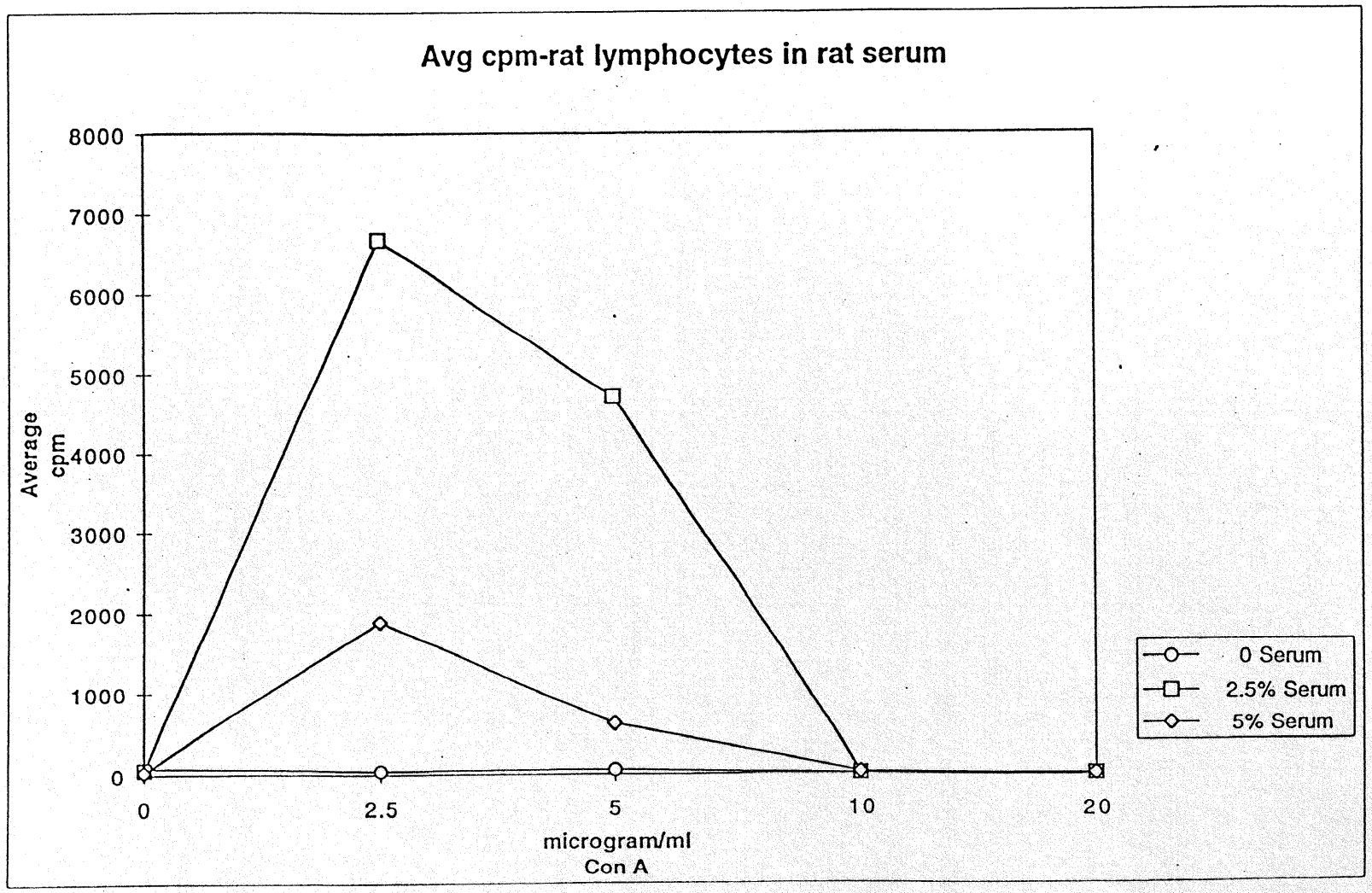


Figure 11. Rat spleen lymphocyte stimulation, cells cultured in RPMI-1640 supplemented with rat serum.

tests after our first lysosome isolation. All samples were reasonably close to those predicted by Trouet (data not shown). In fact, our lysosomes had a 3 fold higher specific activity then expected. This was most likely a result of more efficient instruments in our lab as compared to those used 25 years ago by Trouet. Confident that our procedure worked, only the purity of lysosomes from future isolations was checked.

Hydrolysis experiments similar to those in rat blood were set up to measure the release of MP from MPS (10 μg/ml), and MPS and MP from DexMPS (100 μg/ml) after incubation in isolated lysosomes. Preliminary studies showed no hydrolysis in 3 hours, so others were allowed to incubate for 12 and 24 hours. No significant difference in hydrolysis between drug/lysosome solutions and drug/sucrose solutions samples was found, but hydrolysis was observed. Since the amount of drug released in 24 hours was not double that released in 12 hours, enzyme efficiency *in vitro* was questioned, leading to continued studies at 12 hours. Efforts to increase hydrolysis of MPS and DexMPS in lysosomal contents included sonification of lysosomes to ensure release of their contents; use of fresh lysosomes rather than those frozen at -80°C; fresh lysosomes dialyzed against phosphate buffer to remove sucrose; fresh lysosomes that were filter centrifuged to remove low MW contaminants (sucrose); and lysosomes obtained through a procedure not involving Triton WR-1339 injection (30). Also included were experiments using acetate buffer, citrate buffer, acetate buffer plus Triton X-100 (15), citrate buffer plus EDTA (31), and citrate plus Triton X-100 to dissolve the DexMPS and MPS. In all cases, we were unable to show any difference between incubation in lysosomes and buffer.

Isolation of lymphocytes from rat liver and mitogen stimulation assay

The use of rat serum rather than fetal calf serum had 2 major effects. First, it shifted the peak of the dose-response curve from 10 μg/ ml to 2.5 μg/ ml Con A. Also, the rat cells were able to incorporate more radioactive thymidine in the presence of rat serum than fetal calf serum, indicating that more replication occurred. These two factors indicate that the proliferative ability of the isolated lymphocytes is better determined by growth in rat serum supplemented RPMI than fetal calf serum supplemented RPMI. These experiments show that rat spleenocyte isolation using centrifugation and the density gradient material Histopaque is readily accomplished. Optimum conditions for mitogenic stimulation of the isolated cells consist of 2.5 μg/ ml Con A, and RPMI supplemented with 2.5% rat serum.

Liver cell isolation

During the repeated centrifugation and washing steps the viability of the hepatocytes declines, partly due to the time needed to complete the steps. Fortunately, viability is not essential when measuring the cellular concentration of a large molecule like DexMPS.

Final conclusions

One experiment which can be set up using these procedures involves the administration of DexMPS or MP to rats, followed by their sacrifice at various times from 0 to 96 hours later. With the harvesting and culture of spleen lymphocytes, the effectiveness of the immunosuppressant can be measured. The MP effect should be overcome within 24 hours (26), while the DexMPS effect is hypothesized to remain for several days.

A second experiment could involve the harvesting of liver cells. The liver cell

isolation procedure can be applied to groups of rats sacrificed at 12 and 96-168 hours after DexMPS injection. These two time points would allow any change in the Kupffer cell: hepatocyte ratio to be seen. In addition to observing the ratio of cell populations, the isolated cell suspensions may be able to be substituted for plasma in the HPLC assay, allowing the presence of DexMPS, MPS, or MP to be determined.

Together these procedures provide information concerning hydrolysis of a prodrug in experimental situations. They begin to examine the question of dextran conjugate stability and release of an active agent, and provide a base for future work regarding the long term effects and benefits of targeted drug delivery to the liver. Work yet to be completed concerning administration of DexMPS to rats and the resulting local immunosuppression over time will reveal even more of this interesting puzzle.

In addition, a novel HPLC assay was developed. It is extremely useful for analysis of DexMPS breakdown into MPS and MP, but can also be used for analysis of MPS breakdown to MP. It also has the potential to be modified for similar corticosteroid prodrug systems.

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